

ACE INHIBITORY PEPTIDES FROM PLANT MATERIALS

Field of the Invention

This invention relates to improved processes for obtaining ACE
5 inhibitory peptide compositions with high specific activity from plant sources.

Background of the Invention

Hypertension, or high blood pressure, is a common health problem; in
North America, one in four adults has hypertension. Since hypertension is
10 asymptomatic, irreversible cardiovascular complications may have arisen before
the hypertension is even recognised.

One enzyme which plays a key physiological role in the regulation of
blood pressure is angiotensin converting enzyme (ACE: peptidyldipeptide
hydrolase, EC 3,4,15,1). ACE is involved in increasing blood pressure both
15 through the renin-angiotensin system, by virtue of its ability to convert the
inactive decapeptide angiotensin I to the vasoconstrictive and salt-retaining
peptide angiotensin II (Skeggs et al., 1956) and through the kallikrein-kinin
system, by virtue of its ability to inactivate the vasodilator and natriuretic
nonapeptide, bradykinin (Yang et al., 1970).

20 Inhibition of ACE activity therefore provides a means of lowering blood
pressure.

The first potent and specific inhibitors of ACE activity were discovered by
Ferreira (1965), who showed that an extract of the venom of the Brazilian
arrowhead viper, Bothrops jararaca, potentiated smooth muscle contraction,
25 caused hypotension and increased the capillary permeability induced by
bradykinin. The so-called 'bradykinin potentiating factors' (BPF's) were later
isolated from a number of snake venoms and found to be short peptides which
are potent ACE inhibitors.

A class of synthetic ACE inhibitors has also been made and marketed
30 commercially, the first being captopril (D-2-methyl-3-mercaptopropanoyl-L-
proline). Although many drugs of this group have been found to be invaluable
for lowering high blood pressure, their prolonged use can be accompanied by

unwanted side effects. There therefore remains a need for new therapeutic agents to control hypertension.

It has been known for some time that ACE inhibitory peptides can be obtained by proteolytic digestion of proteins from various sources, including fish
5 (eg. EP 1094071), animal milk proteins (eg. WO 99/65326) and plants (eg. Kawakami et al., (1995); Yano et al., (1996); Pedroche et al., (2002); Wu et al., (2002)). Most of the methods described for obtaining ACE inhibitory peptides from plant proteins involve preliminary purification of the protein before hydrolysis, which adds to the cost and complexity of the process and therefore
10 to the cost of the product.

There remains a need for improved processes for preparing ACE inhibitory compositions from plant materials and for further sources of ACE inhibitory peptides of high specific activity.

15 **Summary of the Invention**

In accordance with one embodiment of the invention is a process for preparing an angiotensin converting enzyme (ACE) inhibitory peptide-containing hydrolysate comprising
; contacting a substantially oil-free seed meal or a flour with an organic
20 solvent,
; separating the meal or flour from the solvent, and
; treating the meal or flour with at least one proteolytic enzyme to
produce an ACE inhibitory peptide-containing hydrolysate.

In accordance with a further embodiment is a process for preparing an
25 ACE inhibitory peptide-containing hydrolysate from flax or canola comprising
; treating a substantially oil-free flax seed meal or a substantially oil-free
canola seed meal with at least one proteolytic enzyme to produce an ACE
inhibitory peptide-containing hydrolysate.

In accordance with a further embodiment is an ACE inhibitory peptide-
30 containing hydrolysate prepared by a process as described above.

In accordance with a further embodiment is an ACE inhibitory peptide-containing hydrolysate produced by partial proteolytic digestion of a flax meal or a canola meal.

In accordance with a further embodiment is a powder produced by drying a hydrolysate in accordance with the invention.

In accordance with a further embodiment is an edible food product comprising a hydrolysate in accordance with the invention.

5 In accordance with a further embodiment is a pharmaceutical composition comprising at least one of peptides Val-Ser-Val and Phe-Leu and a pharmaceutically acceptable carrier.

In accordance with a further embodiment is a peptide of the formula Val-Ser-Val.

10 In accordance with a further embodiment is a peptide of the formula Phe-Leu.

In accordance with a further embodiment is a method of inhibiting ACE activity in a mammal comprising administering to the mammal an effective amount of a hydrolysate or powder or edible product or composition in accordance with the invention.

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Such inhibition of ACE activity can be used to produce a lowering of elevated blood pressure in the mammal. The invention thus provides a method and compositions for treating elevated blood pressure in a mammal, including a human subject.

20 The invention further provides use of a hydrolysate or powder or edible product or composition in accordance with the invention for preparation of a medicament for the treatment of elevated blood pressure in a mammal, including a human subject.

25 **Summary of the Drawings**

Certain embodiments of the invention are described, reference being made to the accompanying drawings, wherein:

Figure 1 shows ACE inhibitory activity (Y axis) as a function of incubation time (X axis), for various proteolytic enzymes acting on canola meal.

30 Figure 2 shows degree of hydrolysis (DH-Y axis) as a function of incubation time (X axis), for various proteolytic enzymes acting on canola meal.

Figure 3 shows absorbance at 214 nm (mAU) in fractions obtained from Sephasil Peptide C₁₈ 12μ ST4.6/250 column.

Detailed Description of the Invention

5 The present invention provides improved processes and new sources for preparing an ACE inhibitory composition from a plant material such as a meal or a flour without the need to first isolate a highly enriched or purified protein fraction from the plant material.

10 As used herein, a "meal" means the non-oil portion of oilseeds after oil extraction, in ground form, and a "flour" means the ground seed of a non-oil producing plant such as a cereal or a legume. The processes of the invention may be applied, for example, to oilseed meals produced by conventional methods such as flaking or expelling an oil seed and defatting and solvent extraction of the resulting meal and to flours obtained from non-oil seeds, 15 such as cereals, pseudocereals and legumes.

A "hydrolysate" as used herein means the digestion mixture obtained after proteolytic digestion or partial proteolytic digestion of a seed meal or flour.

20 In accordance with one embodiment of the invention, the inventors have found that extracting a seed meal or flour with an organic solvent, prior to proteolytic digestion to produce ACE inhibitory peptides, provides hydrolysates with increased ACE inhibitory activity. By using the organic solvent extraction step, the inventors have been able to overcome problems of low yield and low ACE-inhibitory activity which can be encountered when 25 plant material is subjected to direct proteolytic digestion. Table 1 shows that for most plant materials tested, organic solvent extraction of the meal or flour prior to proteolytic digestion increased IC50 and protein content of the hydrolysate.

30 The plant material to be processed may be obtained from an oil-seed, such as flax, canola/rapeseed, soybean, cottonseed, sunflower, peanut or mustard, from a legume seed, such as soybeans, peas, lentils, beans or chickpeas or from a cereal grain such as wheat, oats, barley or rye or a pseudocereal such as buckwheat.

For oil seeds such as canola, flax and soybean, substantially oil-free seed meal is obtained by subjecting the seed to conventional defatting or oil-extraction methods such as expelling, expelling with solvent extraction and flaking and solvent extraction, as practiced in the commercial vegetable oil industry. The resulting material usually contains less than 1% fat. Seeds such as cereal and pseudocereal grains (eg. wheat, oats, rye, buckwheat) are naturally substantially oil free and do not require defatting.

The ground plant material can be in the form of grits obtained by coarsely grinding the whole seed or defatted meal, followed by screening to give particles classified as coarse (10-20 mesh), medium (20-40 mesh) or fine (40-80 mesh) grits according to size, or the ground plant material may be in the form of flour produced by grinding to very fine particles, so that 97% of the product passes through 100-mesh screen. The hull, husk and bran which are generally low in protein may be removed prior to production of the flour.

Suitable organic solvents for extraction of the meal or flour include the lower alcohols such as methanol, ethanol, propanol and butanol, or acetone or ethyl acetate. Ethanol is preferred. The alcohols may be used alone or as an aqueous mixture containing at least 50% alcohol. 65% to 75% ethanol is preferred.

The organic solvent may be mixed with the seed meal or flour at a liquid: solid ratio in the range of about 8:1 to about 25:1, about 10:1 to about 20:1 being preferred.

The seed meal or flour is kept in contact with the organic solvent for about 1 hour up to about 24 hours, at a temperature from about 20°C to the boiling point of the organic solvent. In one embodiment, solvent treatment is for 24 hours at room temperature (25°C), or a shorter time at a higher temperature, eg. 3 hours at 50°C. The meal/solvent slurry may be stirred or otherwise agitated during the contact period.

The treated meal or flour is then separated from the liquid solvent phase by any suitable conventional method, such as centrifugation, screening, filtration or decantation and may be washed with water to reduce the level of residual organic solvent. The material is then ready for proteolytic digestion to generate ACE inhibitory peptides. Alternatively, the residual

organic solvent may be removed by hot air drying to produce a meal or flour that can be stored for future rewetting and proteolytic digestion.

Proteolytic digestion of the seed meal or flour to yield ACE inhibitory peptides may be carried out generally by methods known to those of skill in the art, and as further described herein. Suitable proteolytic enzymes include acid, neutral and alkaline proteases, and peptidases, including serine endopeptidases and metallo endopeptidases, or mixtures thereof. Many commercially available proteolytic enzymes can be used, such as those listed in Table 2. One of skill in the art can readily determine the appropriate digestion conditions for the particular proteolytic enzyme employed.

It is also within the skill of one in the art to determine which proteolytic enzyme gives the lowest ACE inhibitory IC50 in the hydrolysate for a particular plant material, as described in the examples and in Figure 1. It has been found, for example, that thermolysin gives the best inhibitory activity of the enzymes tested when used for digestion of flax seed meal and soybean meal. Proteolysis may be carried out for a period of time which yields the lowest ACE inhibitory IC50 in the hydrolysate. Generally, a digestion period of about 3 hours provides maximum inhibitory activity.

If the hydrolysate is to be used as an edible product such as a food or a food supplement, it may be desirable to stop the proteolytic digestion before maximum ACE inhibitory activity is achieved, in order to retain some of the food value of the plant protein. With some plant materials, proteolytic digestion may be accompanied by the production of bitter-tasting digestion products. In such a situation, it may also be desirable to stop proteolytic digestion before maximum ACE inhibitory activity is reached to control the bitterness.

In one embodiment, the hydrolysates of the invention have an ACE inhibitory IC50 of less than 200 µg powder/ml. In further embodiments, the hydrolysates have an IC50 less than 100, or less than 60 or less than 50.

Typically, the proteolytic enzyme is used at a concentration from about 0.25% to about 8.0% w/w (enzyme:protein content).

In a further embodiment, the proteolytic enzyme is used at of concentration from about 0.5% to about 4.0%.

The proteolytic digestion is terminated by any suitable method, for example heat inactivation of the enzyme or adjustment of the pH of the digestion mixture away from the pH range of enzyme activity. Such methods are well known to those in the art. The resulting hydrolysate is filtered or 5 centrifuged, for example in a decanter centrifuge, to remove residual meal or flour.

In accordance with a further embodiment of the invention is provided a process for preparing an ACE inhibitory peptide-containing hydrolysate from flax or canola meal by digesting the meal with a proteolytic enzyme to 10 generate ACE inhibitory peptide-containing hydrolysates of high specific activity without further purification. Flax and canola have not previously been shown to be sources of ACE-inhibitory peptides. The proteolytic enzyme is selected; and the digestion carried out, as described above. The flax or canola meal may optionally be subjected to extraction with an organic solvent, 15 as described above, before proteolytic digestion.

After digestion of a seed meal or flour to give ACE-inhibitory peptides, and separation of the hydrolysate from the residual meal or flour, the hydrolysate can be used as an edible product or may be spray dried to give a water-soluble powder suitable for use as an edible product or as a 20 pharmaceutical. The use of protein and hydrolysed protein preparations as edible products and their incorporation into food products or use as food supplements is well known to those of skill in the food processing art, for example as described in Clemente, A (2000) "Enzymatic Hydrolysates in Human Nutrition", in Trends in Food Science & Technology v. 11, pp. 254-262

25 In one embodiment, the hydrolysates are used as edible products without further processing other than standard procedures to control bacterial contamination, such as flash pasteurisation or microfiltration.

The hydrolysates of the invention in liquid or powder form may be used to supplement beverages such as soft drinks, carbonated beverages, ready to 30 mix beverages, milk and milk beverages and their derivates, and foods such as sauces, condiments, salad dressings, fruit juices, syrups, desserts (e.g., puddings, gelatin, icings, and fillings, baked goods and frozen desserts such as ice creams and sherbets), soft frozen products (e.g., soft frozen creams,

soft frozen ice creams and yogurts, soft frozen toppings such as dairy or non dairy whipped toppings), oils and emulsified products (e.g., shortening, margarine, mayonnaise, butter and salad dressings), candy and bar confections, cereal foods, and chewing gum tablet.

5 It has long been realised that extremely raised blood pressure is a life threatening condition. It has more recently been suggested that even modest increases in blood pressure above normal may have deleterious effects.

10 A systolic blood pressure of 140 mm of mercury or greater, (occurring in approximately 20% of the adult population over the age of 30) is the current clinical definition of hypertension requiring medical intervention. A number of epidemiologists are, however, now defining a second or "preventative model" of hypertension which would define a systolic blood pressure of more than 120 mm of mercury as being the point at which people should undertake steps to reduce their blood pressure and associated risk of cardiovascular disease. People who have blood pressure within the range 120-140 mm mercury generally do not consider themselves to be sick and therefore are less likely to receive pharmaceutical based therapies. An increasing percentage of people in this category are, however, seeking non-pharmaceutical options to reduce this moderately elevated blood pressure

15 (Potential benefits of functional foods and nutraceuticals to reduce the Risk and Costs of Diseases in Canada. B.J. Holub. Report to AAFC 2002).

20 The hydrolysates and powders of the invention provide anti-hypertensive agents which may be ideal for such cases of modestly raised blood pressure and can be consumed along with or as part of a food.

25 By comparison with the blood lowering effects previously observed in humans for ACE inhibitory products derived from milk or whey, the hydrolysates of the invention with IC50 values around 40-60 μ g/ml may be given to human subjects at an initial dose from about 2 to about 5 gm per day. This dose may be adjusted as required once its blood pressure lowering effect

30 is observed. The dosage would be adjusted accordingly for hydrolysates of higher or lower IC50 value, as would be understood by one of skill in the art.

The hydrolysates may also be formulated as tablets, capsules, granules, powders, syrups, suspensions or injectable solutions.

The hydrolysates produced by the methods described herein have equivalent or greater ACE inhibitory activity than the commercially available whey hydrolysates. When analysed by the ACE inhibitory assay described herein, the commercial whey product BioZate (Davisco Foods International) 5 had an ACE inhibitory IC₅₀ of 137 µg powder/ml. As shown herein, several plant materials yielded protein hydrolysates with ACE inhibitory IC₅₀ values considerably less than that of the whey product, and therefore greater inhibitory activity, without the need to employ any subsequent purification strategy.

10 Hydrolysates obtained by proteolytic digestion of canola meal were further purified, as described in Example 12, to give two fractions, each containing a single purified ACE-inhibitory peptide. These peptides, not previously described as ACE inhibitors, have the amino acid sequences Val-Ser-Val and Phe-Leu.

15 The ACE inhibitory peptide-containing hydrolysates of the invention may be further processed to obtain fractions with further enhanced specific activity or the purified single peptides described above, for use as edible products or as pharmaceuticals.

20 The ACE inhibitory activity of the hydrolysates of the invention may be further enhanced, if desired, by ultrafiltration as described in the Examples herein.

25 It has been found that seeds with a high polysaccharide content, such as flax and barley, give hydrolysates of high viscosity. Where the viscosity of the hydrolysate is greater than about 10×10^{-3} PaS (based on the viscosity of a 5 % solution in water at 25°C and a shear rate of 200 (l/s), ultrafiltration membranes up to pore size 100,000 MWCO can be used, but membranes with pore size up to 10000 are preferred.

30 For hydrolysates of lower viscosity, such as those obtained from canola, membranes of pore size up to 10000 MWCO may be used and membranes with pore size up to 3000 MWCO are preferred.

EXAMPLES

The examples are described for the purposes of illustration and are not
5 intended to limit the scope of the invention.

Methods of chemistry, molecular biology, protein and peptide
biochemistry and immunology referred to but not explicitly described in this
disclosure and examples are reported in the scientific literature and are well
known to those skilled in the art.

10

Reagents

Chemicals and enzymes were obtained as follows: angiotensin
converting enzyme (from rabbit lung) and HHL (Sigma Chemical Co., St.
Louis, MO, USA); HA and trifluoroacetic acid (TFA) (Acros Organics, New
15 Jersey, USA); HPLC grade acetonitrile (Fisher Scientific, Nepean, ON,
Canada). All other chemicals were of reagent grade and also obtained from
Fisher Scientific. HPLC-grade water was generated by a Milli-Q system
(Millipore, Bedford, MA, USA).

20

Methods

The determination of ACE-inhibitory activity

The *in vitro* antihypertensive activity (ACE-inhibitory activity) was measured
by a new improved HPLC method (Wu, J. P. et al., (2002)), modified from the
method of Cushman and Cheung (1971). This method utilizes reverse phase
25 high performance liquid chromatography (HPLC) to separate and quantify the
ACE-catalyzed production of hippuric acid (HA). Hippuryl-histidyl-leucine
(HHL) and HA were separated on a Symmetry C₁₈ column by gradient elution
that used mixtures of trifluoroacetic acid (TFA)/acetonitrile and TFA/water as
solvents. In comparison to the standard spectrophotometric method, the new
30 HPLC method eliminates the need for ethyl acetate extraction of HA and
allows direct injection of ACE reaction mixtures onto the HPLC column.

Sample preparation for HPLC analysis

For direct HPLC analysis, the total reaction volume was 70 μ l, composed of 50 μ l of 2.17 mM HHL, 10 μ l of 2 mU of ACE and 10 μ l of different concentrations of protein hydrolysates (all prepared with 100 mM borate buffer, containing 300 mM NaCl, pH 8.3). For the control test, 10 μ l of buffer solution was used. The HHL and protein hydrolysates or the control buffer solutions, were combined and maintained at 37°C for 10 min in 1.5 ml polyethylene micro-centrifuge tubes. ACE was also maintained at 37°C for 10 min before the two solutions were combined and incubated at 37°C in an Eppendorf Thermomixer R (Brinkmann Instruments, Inc. New York, USA) with continuous agitation at 450 rpm. The reaction was terminated after 30 min by addition of 85 μ l of 1 N HCl and the solution filtered through a 0.45 μ m nylon syringe filter for reverse phase RP-HPLC (RP-HPLC) analysis.

15 High-performance liquid chromatography

HPLC was performed on a 2690 Separation Module equipped with a 996 Photodiode Array Detector (Waters Inc, MA. USA). Instrument control, data collection and analysis were undertaken using Millennium Chromatography Manager Software version 2.15 (Waters Inc, MA. USA). Samples (10 μ l) were analyzed on a Symmetry C18 column (3.0 x 150 mm, 5 μ m, Waters Inc., MA, USA) and HA and HHL were detected at 228 nm. The column was eluted (0.5 ml.min⁻¹) with a two solvent system: (A) 0.05% trifluoroacetic acid (TFA) in water and (B) 0.05% TFA in acetonitrile, with a 5-60 % acetonitrile gradient for the first 10 min, maintained for two min at 60% acetonitrile, then returned to 5% acetonitrile for one min. This was followed by isocratic elution for four min at the constant flow rate of 0.5 ml.min⁻¹. External standard HA samples were prepared freshly and used for calculation of the concentration of HA formed by the action of ACE in the presence of protein hydrolysates (HA_{hydrolysate}) or in the absence of protein hydrolysate (HA_{control}), blank sample was prepared as: the ACE was inactivated first by the addition of HCl before the incubation.

ACE inhibitory activity calculation

Inhibitory activity was calculated according to the following equation:

5 Inhibitory activity (%) = [(HA_{hydrolysate} - HA_{control}) / (HA_{control} - HA_{blank})] × 100

The IC₅₀ value was defined as the amount of inhibitory substance in the reaction mixture that resulted in 50% inhibition of ACE activity, measured as described above. A plot of inhibition activity (%) versus concentration of 10 protein hydrolysate (µg powder/ml) was generated using at least 5 different concentrations of the same hydrolysate sample. Regression was conducted with the Microsoft Excel 97 SR-1 software (Microsoft Corporation).

Determination of Degree of Hydrolysis (DH) of Proteins

15 Under alkaline or neutral conditions, the pH was monitored and maintained constant by addition of base. The amount of base added was recorded at set time intervals and used to calculate the degree of hydrolysis (DH). The DH is calculated from the volume and molarity of base to maintain 20 constant pH and is expressed as the percent ratio of numbers of peptide bonds broken (h) to the total numbers of bonds per unit weight according to the formula of Adler-Nissen (1986).

Under acid hydrolysis conditions, the degree of hydrolysis (DH) was determined according to the method of Adler-Nissen J. (1979).

25

Hydrolysate sample preparation

30 Samples of hydrolysis reaction mixtures were drawn at various time intervals and quickly transferred to test tubes. From each sample, 2.0 ml aliquots were rapidly pipetted into each of two test tubes containing 10 ml of 1% NaDODSO₄, and the test tubes were kept at 75°C by immersing in a water bath with shaking for at least 15 min to disperse the protein hydrolysate.

The contents of each test tube were transferred quantitatively to a 50-ml volumetric flask and diluted to volume with 1% NaDODSO₄. The content of free amino groups, expressed as leucine amino equivalents, was assayed by the TNBS reaction.

5

TNBS Reaction

0.25 ml prepared sample or blank (1.0% SDS) or standard solution was mixed with 2.0 ml of phosphate buffer at pH 8.2.

- 10 2 ml of 0.1% TNBS solution was added and the test tube was shaken and placed in 50°C bath for 60 min, covered by aluminum foil.
- 4.00 ml of 0.1 N HCl was added to terminate the reaction and the absorbance was read against water at 340 nm after 30 min.
- 15 The DH was calculated using the modified method of Beak et al. (1995) as follows:

$$DH = (L_t - L_0) / (L_{max} - L_0) \times 100$$

- 20 where L_t is the amount of α -amino acid released at time t ; L_0 is the amount of α -amino acid in original canola solution; and L_{max} is the maximum amount of α -amino acid in canola meal after acid hydrolysis (conducted under 6N HCl at 100°C for 24 h).
- 25 **Example 1. Enzymatic screening for the production of ACE inhibitory peptides (ACEIP) from defatted canola meal**

The reaction was performed in batches in a reaction vessel equipped with a stirrer, thermometer, and pH electrode. Solvent extracted expeller cake 30 commercial canola meal (Canbra Foods, Lethbridge, Alberta, Canada), (31.16g), ground to pass a 40 mesh screen (protein content: 40.1%), was mixed thoroughly using a magnetic stirrer with distilled water to form 250 ml of

a 5 % protein slurry. The proteolytic enzymes evaluated are listed in Table 2, along with their optimum pH and temperature conditions. The pH of the slurry was adjusted to the appropriate pH and temperature for the enzyme being tested. Enzyme (4 %, w/w on the basis of protein content of slurry) was 5 added to initiate the reaction. The pH of the reaction was maintained at a constant value by addition of either 0.5 N NaOH or 1 N HCl. The volumes of alkali were recorded at specific intervals for the calculation of degree of hydrolysis (DH).

10 Samples were taken at 30, 60, 120, 180, 300 min intervals and enzyme was inactivated by heating in boiling water for 15 min. The pH of the slurry was then adjusted to pH 4.0 to precipitate unhydrolysed proteins, large peptides and all solids. The precipitate was removed by centrifugation at 10,000 × g for 25 min. The resulting clear supernatants were then freeze dried and stored at -5°C till further analysis. A control (without proteolytic enzyme) 15 sample was also generated using the same procedure. The freeze dried supernatants were then evaluated for their ACE inhibitory activity.

16 The ACE inhibitory activity (%) of canola protein hydrolysates digested by different enzymes is shown in Fig 1. Results indicated that ACE inhibitory activity (%) varied greatly, from 0 % to 50.5 %, with the average value of 28.4 ± 13.47 %. Alcalase 2.4 produced the most potent ACE inhibitory hydrolysate from canola meal. Other enzymes which generated significant ACE inhibitory activity included Protease S, Protease M, alkaline protease and neutral protease. Although not included in this study, Thermolysin was subsequently determined to produce a peptide fraction with similar ACE inhibitory activity to 25 that observed for Alcalase 2.4 hydrolysates. Generally, any proteolytic enzyme which, in 3 hours digestion, generates a hydrolysate giving >35 % ACE inhibition is preferred. No ACE inhibitory activity was detected for the unhydrolysed control canola meal samples, indicating that it was the breakdown of canola protein by the action of proteolytic enzymes that resulted 30 in the formation of ACE inhibitory peptides. When the incubation time for Alcalase 2.4 and Protease M was extended to 24 hours there was no significant increase in the ACE inhibitory activity beyond 5 hours of incubation.

The relationship between degree of hydrolysis (DH) and incubation time is shown in Fig 2; DH increased with increased incubation time. Alkaline Protease and Alcalase 2.4 gave the highest DH values. Protease M and Protease S enzyme treatments had moderate DH values, while their corresponding hydrolysates showed relatively high ACE inhibitory activity. Pepsin, Chymotrypsin and trypsin had the lowest DH values, which indicated that these enzymes had very limited ability to hydrolyse canola protein. There was no positive relationship between DH value and ACE inhibitory activity.

Sequential treatment of canola meal with different hydrolytic enzymes was investigated. After three hours of hydrolysis with Alcalase 2.4, the pH and temperature of the solution was adjusted to the optimum conditions for each of the four other enzymes that produced hydrolysates with high ACE inhibitory activity. The second enzyme was then added and the solution incubated for a further 3 hours. Of the protein hydrolysates resulting from sequential hydrolysis with two different enzymes, only the product derived from sequential hydrolysis with Alcalase 2.4 and Alkaline Protease had a significantly improved IC₅₀ value (40.0 µg powder/ml) over the activity of the single enzyme product (Alcalase 2.4 = 68.6 µg powder/ml), or any of the other proteases by themselves (55.7-78.6 µg powder/ml).

20

Example 2. Preparation of flaxseed meal enzymatic hydrolysates

The reaction was performed in batches in a reaction vessel as in Example 1. Solvent extracted expeller cake commercial flaxseed meal (CanAmara Foods, Atton, Manitoba, Alberta) (50 g), ground to pass a 40 mesh screen (protein content: 33.9%), was mixed thoroughly using a magnetic stirrer with distilled water into a 750 ml slurry. The pH of the slurry was adjusted to 8.0 and temperature to 60°C for Alcalase 2.4 enzyme hydrolysis. The enzyme was added at a ratio of 4 % (w/w, on the basis of protein content of slurry). During the reaction, the pH of the slurry was maintained at a constant value by addition of 0.5 N NaOH. The enzyme was inactivated by adjusting the pH to around 4.0 with 6 N HCl. Unhydrolysed proteins, large peptides and insoluble material were removed by centrifugation at 6,000 ' g for 25 min. The residue was resuspended in 250 ml

water and centrifuged under the same conditions. The resulting clear supernatants were then combined, freeze dried and stored at -5°C till further analysis. At the same time, the control extract (without enzyme) was prepared under the same conditions. The hydrolysate yield was 53.54% (by weight) 5 with a protein content of 39.9%, and an ACE inhibitory IC50 of 64.3 µg powder/ml.

A range of proteolytic enzymes was then evaluated, as in Example 1; results with flax were similar to those found with canola meal.

10 **Example 3. Production of ACE inhibitory peptides from Soy flour.**

Nutrisoy 7B soy flour (87g, Archer Daniels Midland CO., Decatur, Illinois) was mixed thoroughly using a magnetic stirrer with distilled water into 600 ml slurry. The slurry was adjusted to pH 8.0 and 60°C for enzymatic hydrolysis by Acalase 2.4 as in Example 2. Enzyme was added at the ratio of 15 4 % (w/w, on the basis of protein content of slurry). During the reaction, the pH of the slurry was maintained constant with 0.5 N NaOH. The enzyme was inactivated by adjusting the pH to around 4.0 with 6 N HCl. Unhydrolysed proteins and large peptides as well as other polymers were removed by centrifugation at 6,000 x g for 25 min. The residue was resuspended in 300 ml 20 water and centrifuged using the same conditions. The resulting clear supernatants were then combined and freeze dried and stored at -5°C till further analysis. The hydrolysate produced by Alcalase 2.4 had an ACE inhibitory IC50 of 126.3 µg powder/ml with a product yield of 82.8% W/W.

25 **Example 4. Production of ACE inhibitory peptides from pea flour and oat flour**

Pea flour and oat flour (Parrheim Foods, Saskatoon, Canada) were incubated with Alcalase 2.4 under the same conditions as described in Example 2. The oat flour hydrolysate had the weakest ACE inhibitory activity 30 (1200 µg powder/ml) of all the plant materials tested. Pea flour had an ACE inhibitory activity of IC50 87.1 µg powder/ml.

Example 5. Production of ACE inhibitory peptides from whole buckwheat meal

Buckwheat was purchased from a local grocery store, ground and passed through a 40 mesh screen. Samples of the meal were hydrolysed 5 with alcalase 2.4 L or Thermolysin, as described in Example 2. The protein hydrolysate produced by Thermolysin had an ACE inhibitory IC₅₀ of 78.6 µg powder/ml with a product yield of 18 %, while the ACE inhibitory IC₅₀ of the Alcalase 2.4 L-treated hydrolysate was 174.3 µg powder/ml, with a product yield of 37.1 %.

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Example 6. Production of ethanol-treated canola meal ACE Inhibitory hydrolysate

Defatted commercial canola meal as in Example 1 (31.2g), ground to pass a 40 mesh screen, was extracted with 70 % (v/v) ethanol:water solution 15 at a ratio of 1:10 (weight/volume), with agitation for 12 hours at room temperature or at 50°C for 3 hours. The meal was recovered, washed with distilled water and subjected to hydrolysis by Alcalase 2.4, Protease M, Protease S, or a sequential treatment of Alcalase 2.4 and Alkaline Protease, for a period not exceeding 3 hr per enzyme (combined incubation for total 6 20 hours). The ethanol-treated canola meal enzymatic digests were then fractionated by centrifugation to produce a supernatant containing the hydrolyzed peptides and a precipitate which contained the unhydrolyzed proteins and residual meal. These hydrolysates show ACE inhibitory activity (IC₅₀) from 40.0 µg powder/ml to 64.3 µg powder/ml (Table 3). The protein 25 content of the hydrolysates produced from ethanol-treated meal was significantly increased, from 41.7% to 54.6-60.4 %, except for the hydrolysate resulting from incubation with Protease S where the protein content was only 28.7%.

Example 7. Preparation of ethanol-treated defatted flaxseed meal enzymatic hydrolysates

Defatted flaxseed meal as in Example 2 (50 g), ground to pass a 40 mesh screen (protein content: 33.9%), was treated with 70% (v/v) ethanol: water solution (1/15. w/v) at 50°C for 3 hours. The extracted flaxseed meal

was recovered by filtration and dispersed in water to form a 750ml water slurry using a magnetic stirrer. The production of enzymatic hydrolysate with Alcalase 2.4 was performed as described as in Example 2. The ethanol-treated defatted flaxseed hydrolysate had a yield of 52.0% based on product 5 weight, with a protein content of 45.5% and the ACE inhibitory IC50 was 51.4 µg powder/ml.

Defatted flaxseed meal recovered after the extraction of lignan (SDG) as reported by Westcott & Muir (U.S. Pat. No 5,705,618), was also used for the production of ACE inhibitory hydrolysates. The protein hydrolysate 10 generated by Alcalase 2.4 treatment of this material had a similar ACE inhibitory activity (IC50 = 55.7 µg powder/ml), indicating that the recovery of the valuable phytochemical SDG from the meal does not interfere with the ability to produce biologically active peptides from the residual meal.

15 **Example 8. Production of ACE inhibitory peptides from ethanol-treated soy flour using Alcalase 2.4 and/or Thermolysin enzymes**

Nutrisoy 7B soy flour (87g, Archer Daniels Midland CO., Decatur, Illinois) was treated with 70% ethanol for 3 hours at 50°C. The filtered residue was mixed thoroughly using a magnetic stirrer with distilled water into 600 ml 20 slurry. The slurry was adjusted to the appropriate pH and temperature for digestion by Alcalase Acalase 2.4 L or Thermolysin, as in Table 2. The enzyme was added at a ratio of 4 % (w/w, on the basis of protein content of slurry) for Alcalase 2.4 or at 1 % (w/w, on the basis of protein content of slurry) for Thermolysin. During the reaction, the pH of the slurry was 25 maintained constant with 0.5 N NaOH if necessary. The enzyme was inactivated by adjusting the pH to around 4.0 with 6 N HCl. Unhydrolysed proteins and large peptides as well as other polymers were removed by centrifugation at 6,000 x g for 25 min. The residue was resuspended in 300 ml water and centrifuged using the same conditions. The resulting clear 30 supernatants were then combined and freeze dried and storied at -5°C till further analysis. The hydrolysate produced by Thermolysin had an ACE inhibitory IC50 of 42.9 µg powder/ml with a product yield of 64.7%, while

Alcalase 2.4 treatment resulted in a hydrolysate with an ACE inhibitory IC₅₀ of 118.1 µg powder/ml with a product yield of 55.7%.

5 **Example 9. Production of ACE inhibitory peptides from flaxseed using Thermolysin**

Defatted flaxseed meal as in Example 2 (50 g), ground to pass a 40 mesh screen (protein content: 33.9%) was mixed thoroughly using a magnetic stirrer with distilled water into a 750 ml slurry. The pH of the slurry was adjusted to the appropriate pH and temperature for the following enzymes:

10 Thermolysin (39 units/mg solid), or incubation with Alcalase 2.4 for 3 hour and followed by incubation for a further 3 hours with Thermolysin. The enzyme was added at a ratio of 4 % (w/w, on the basis of protein content of slurry) for Alcalase 2.4 or 1 % (w/w, on the basis of protein content of slurry) for Thermolysin. After initiation of the reaction, the pH of the slurry was

15 maintained at a constant value by addition of 0.5 N NaOH if necessary. The enzyme was inactivated by adjusting pH to around 4.0 with 6 N HCl. Unhydrolysed proteins and large peptides as well as other polymers were removed by centrifugation at 6,000 x g for 25 min. The residue was suspended again with 250 ml water and centrifuged using the same

20 conditions. The resulting clear supernatants were combined, freeze dried and stored at -5°C till further analysis. The hydrolysate produced by Thermolysin had an ACE inhibitory IC₅₀ of 37.1 µg powder/ml. The hydrolysate resulting from the sequential hydrolysis of Thermolysin and Alcalase 2.4 had an ACE inhibitory IC₅₀ of 34.2 µg powder/ml. Thermolysin is a more effective enzyme

25 for the production of ACE inhibitory peptides from flaxseed compared to Alcalase 2.4 alone which produced a hydrolysate with an IC₅₀ of 64.3 mg powder/ml.

30 **Example 10. Ultrafiltration processing of Canola ACE Inhibitory peptides**

Defatted canola meal (62.3g) ground to pass a 40 mesh screen was treated with 70% ethanol (v/v), at ratio of 1:10, W/V). After filtration to remove the aqueous alcohol, the residue was further digested by Alcalase 2.4 to produce an ethanol-treated canola protein hydrolysate. The resulting protein

hydrolysate was further purified by ultrafiltration over 10000, 3000, 1000 molecular weight cut off (MWCO) membranes. Ultrafiltration was conducted using an Amicon Ultrafiltration Cell Model 8200 (Amicon Division, W.R. Grace & Co., Beverly, USA) by applying Argon at a pressure of 35 PSI to the

5 solution on the retentate side of the membrane. The peptide hydrolyzate was initially separated into retentate and permeate fractions using a 10000 MWCO membrane. The permeate from the 10000 membrane was then subjected to ultrafiltration over the 3000 MWCO membrane to generate a 3000 MWCO retentate and a 3000 MWCO permeate. The 3000 MWCO permeate was

10 further fractionated by ultrafiltration over a 1000 MWCO membrane to produce a 1000 MWCO permeate and a 1000 MWCO retentate. A second set of experiments were conducted where the total protein hydrolysate was subjected to ultrafiltration over the 3000 MWCO and 1000 MWCO membrane without prior treatment over the 10000 MWCO membrane. Permeates and

15 retentates were collected and freeze dried for ACE inhibitory IC₅₀ determination. All permeates contained peptides with greater ACE inhibitory activity than the corresponding retentate fractions. The highest ACE inhibitory activity (IC₅₀ = 25.7 µg powder/ml) was found in the permeate obtained by direct ultrafiltration of protein hydrolysate over the 1000 MWCO membrane

20 (Table 4). Peptide fractions with high ACE inhibitory activity (IC₅₀ = 30.0-31.4 µg powder/ml) were also obtained by ultrafiltration over the 3000 MWCO membrane, either directly or after ultrafiltration over a 10000 MWCO membrane. Since the 3000 MWCO permeate product yield was higher than obtained with the 1000 MWCO membrane, there was little advantage in

25 proceeding to the 1000 MWCO step. As membrane flux decreases with decreasing pore size, the optimum yield was obtained with the 3000 MWCO membrane.

30 **Example 11. Ultrafiltration processing of flaxseed ACE Inhibitory peptides**

The liquid hydrolysates prepared in Examples 7 and 9 were further purified by ultrafiltration over 10000 and 100000 molecular weight cut off (MWCO) membranes as in Example 10. Ultrafiltration was conducted

individually, both permeates and retentates were collected and freeze dried for ACE inhibitory IC₅₀ determination. As in the previous example, all retentates contain peptides with greater ACE inhibitory activity than those found in the retentate (Table 5). The viscosity of the flaxseed hydrolysate was 5 much greater than that observed for canola protein hydrolysates, therefore it was impracticable to conduct ultrafiltration with membranes of 3000 and 1000 MWCO. When operating with 10 K and 100 K MWCO membranes, difficulties were encountered when conducting ultrafiltration due to the formation of a film on the surface of the membrane. The formation of this film can be avoided by; 10 A) stirring and heating the solution to a temperature greater than 40°C; B) adjusting the pH to pH 3-4; or C) combination of A and B. Compared to the ACE inhibitory IC₅₀ value of the original flaxseed hydrolysate (64.2 µg powder/ml), no significant improvement was achieved in these ultrafiltration steps since, because of the large pore size of the 100K MWCO membranes, 15 large peptides can also pass through the membrane into the permeate. In the case of the ethanol-treated hydrolysate, the permeates had improved IC₅₀ values of 30.0 and 35.7 µg powder/ml, compared to the original value at 51.4 µg powder/ml, indicating that the activity of the ACE inhibitory peptide fraction produced from the alcohol washed defatted flax meal can be improved by 20 passage over either an 10K or a 100K MWCO membrane.

Example 12. Purification and identification of highly bioactive ACE inhibitory peptides from canola protein hydrolysates by reversed phase chromatography

25 Defatted canola meal Alcalase 2.4 hydrolysate prepared as in Example 6 was dissolved in 1% acetic acid water (v/v) solution at a concentration of 100 mg/ml for preparative chromatography. A segmented column composed of three PrepPak 40 mm Cartridges (Bondapack C-18 15-20 µm, 40x100 mm, Waters) and a guard column (40 x 10 mm) were coupled with a PrepLC 30 4000 system (Waters). Instrument control, data collection and analysis were undertaken using Millennium Chromatography Manager software v 2.15 (Waters Inc, MA, USA). The sample (20ml) was injected automatically via solvent delivery system. The absorbance profile was monitored at 280 nm

using a 2487 Absorbance Detector. Fractions were collected automatically using a Waters Fraction Collector. The column was eluted (50 ml.min⁻¹) with a two solvent system: A: water containing 1% acetic acid and B: methanol (100%) (Table 6).

5 All fractions collected were assessed for ACE inhibitory activity. A total of 32.6 g of canola meal Acalase hydrolysate was fractionated by preparative chromatography and the yield of the most active peptide fraction was 2.8 g (8.5%) with an ACE inhibitory IC50 of 2.0 µg powder/ml.

10 The active fraction from the preparative C-18 was further purified by SephasilTM Peptide C18 ST 10/250 column (particle size:12 µm, Pharmacia Biotech, Sweden) coupled with an ÄKTA explorer 10XT system controlled by Unicorn system (Pharmacia Biotech, Sweden). The column was eluted with a two solvent system: A: 20 mm sodium monobasic phosphate buffer solution (0.05% TFA) and B: 20 mm sodium monobasic phosphate (80% acetonitrile 15 containing 0.05% TFA) at the flow rate of 5 ml/min, after 5 column volume (CV) isocratic elution at 5% B, gradient elution from 5% B- 20% B in 15 CV. The injection volume was 500 ml of a 200 mg/ml solution of the most active preparative chromatography fraction. The absorbance was monitored at 214 nm and fractions were collected. A total of 21 fractions were collected and the 20 most potent fractions were F₁₃ and F₁₈. After repeat injection, 1.30 g of F₁₃ and 0.85 g of F₁₈ with ACE inhibitory (IC50 values of 38.6 and 312.9 µg powder/ml respectively) were obtained. These IC50's were higher than the starting material due to the presence of the non-volatile butters used in the chromatographic separation.

25 Each of F₁₃ and F₁₈ were re-chromatographed on a SephasilTM Peptide C₁₈ ST 10/250 column (particle size:12 um, Pharmacia Biotech, Sweden) using a two solvent system: A water (containing 0.1% TFA) and B acetonitrile (containing 0.1% TFA) at the gradient of 10% B -30% B in 7 CV at the flow rate of 5 ml/min to de-salt and further purify these active fractions. The major 30 potent fraction peaks were identified as F₁₃₋₁₁ resulting from the chromatography of F₁₃, and F₁₈₋₅ from the chromatography of F₁₈. The yield for F₁₃₋₁₁ was 18.32 mg with an ACE inhibitory IC50 of 0.44 µg/ml, and for F₁₈₋₅ the yield was 4.89 mg with an IC50 value of 0.37 µg/ml.

Fractions F_{13-11} and F_{18-5} were further purified (Figure 3) by Sephasil Peptide C18 12 ST 4.6/250 column (Pharmacia Biotech, Sweden) using a two solvent system; A: water (containing 0.1% TFA) and B: acetonitrile (containing 0.1% TFA) with a gradient of 5% B - 40% B in 10 CV at a flow rate 5 of 1.5 ml/min. The injection volume was 30 ml of a 25mg/ml solution. The absorbance was monitored at 214 nm and fractions were collected.

The identity of the peptides in the purified single peak fractions F_{13-11a} and F_{18-5a} from the ST 4.6/250 column were determined by atmospheric pressure chemical ionization (APCI) mass spectrometry using a Quattro LC 10 liquid chromatograph/Mass Spectrometer (Micromass, UK) equipped with APCI probe, Z-Spray interface, separation module and photodiode array detector (Waters Inc. MA, USA). Instrument control and data analysis were performed using the MassLynx software (Micromass, UK). A peptide sample (10ml) was chromatographed on a Symmetry C₁₈ column (2.0 x 150 mm; 5 15 mm). The sample was eluted (0.3 ml·min⁻¹) with a two solvent system: (A) 0.1% formic acid (FA) in water and (B) 0.1% FA in acetonitrile, with a 5-60 % acetonitrile gradient for the first 10 min, maintained for 2 min at 60% acetonitrile, then returned to 5% acetonitrile for 1 min. This was followed by isocratic elution for 4 min at the constant flow rate of 0.3 ml·min⁻¹. Positive and 20 negative ion intensities were recorded from 50 to 500 m/z with a 1.5 sec scan time. The analyzer vacuum was 2.2e⁻⁵ torr. The two most bioactive peptides were identified as Valine-Serine-Valine and Phenylalanine-Leucine (Table 7). Neither of these two amino acid sequences have previously been reported as having ACE inhibitory activity.

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Table 1

ACE inhibitory IC 50 for alcalase 2.4 digested plant hydrolysates

<i>Raw material</i>	<i>Ethanol extraction</i>	<i>IC50 (µg powder/ml)</i>	<i>Protein content (%)</i>
canola meal	yes	41.4	60.4
canola meal	no	68.6	41.7
flax meal	yes	51.4	45.5
flax meal	no	64.3	39.9
pea flour	yes	87.1	63.5
pea flour	no	131.4	59.3
soy meal	yes	126.3	63.8
soy meal	no	118.1	55.3
oat flour	yes	1198.6	8.3
oat flour	no	1044.3	14.2

Table 2
Hydrolysis of canola meal by various proteolytic enzymes

Common Name	Trade Name	pH	T(°C)	Source
Umamizyme	Umamizyme	8	45	Amano Pharmaceutical Co., Ltd. Japan
Protease A	Protease A "Amano" 2G	7	50	Amano Pharmaceutical Co., Ltd. Japan
Protease P	Protease P "Amano" 6	10	60	Amano Pharmaceutical Co., Ltd. Japan
Peptidase R	Peptidase R	7	45	Amano Pharmaceutical Co., Ltd. Japan
Protease M	Protease M "Amano"	4.5	50	Amano Pharmaceutical Co., Ltd. Japan
Protease S	Protease S "Amano"	8	70	Amano Pharmaceutical Co., Ltd. Japan
Proleather FG-F	Proleather FG-F	10	60	Amano Pharmaceutical Co., Ltd. Japan
Alcalase 2.4L	Alcalase 2.4L	8	60	Novo Nordisk, North America, Inc., North Carolina.
Alkaline Protease	ENZECO Alkaline Protease L-FG	10	50	Enzyme Development Corporation, New York.
Neutral Protease	ENZECO Neutral Protease NBP-L	7	40	Enzyme Development Corporation, New York.
Pepsin	porcine stomach mucosa	2	37	Sigma, St. Louis, MO, USA.
Trypsin	Bovine Pancreas	8	37	Worthington Biochemical Corporation, NJ, USA.
Chymotrypsin	Bovine Pancreas	8	25	Worthington Biochemical Corporation, NJ, USA.
Thermolysin	Thermolysin	8	55	Sigma, St. Louis, MO, USA.

Table 3. ACE inhibitory IC50 and protein content of ethanol-treated canola meal hydrolysates

Enzyme	IC50 (µg powder/ml)	Protein (%)	Product Yield (%)
Protease S	64.3	28.7	19.3
Protease M	40.0	56.4	32.8
Alcalase 2.4	68.6	60.4	38.6
Alcalase 2.4L+ Alkaline Protease	40.0	54.6	45.4

Table 4. ACE inhibitory IC50, protein content and yield of permeates and retentates resulting from the ultrafiltration treatment of canola hydrolysate

	IC50 (µg powder/ml)	Protein content (%)	Product Yield (%)
stepwise operation			
10,000 permeate	37.1	59.3	73.5
retentate	54.3	54.0	20.4
3,000 permeate	30.0	56.3	70.5
retentate	50.0	66.2	28.6
1,000 permeate	30.0	46.9	43.5
retentate	31.4	68.5	47.0
Individual operation			
10,000 permeate	37.1	59.3	73.5
retentate	54.3	54.0	20.4
3,000 permeate	31.4	58.9	73.3
retentate	52.9	57.0	25.2
1,000 permeate	25.7	50.3	56.0
retentate	52.9	68.0	35.7

Table 5. Yield, protein content and ACE inhibitory IC50 of defatted flax meal Alcalase 2.4 L hydrolysates subjected to ultrafiltration.

	<i>IC50 (µg powder/ml)</i>	<i>Protein content (%)</i>	<i>Product Yield (%)</i>
Defatted flaxseed hydrolysate			
100K permeate	48.6	44.5	53.6
100K retentate	54.3	32.1	39.3
10K permeate	48.6	44.8	50.9
10K retentate	61.4	31.6	39.0
EtOH treated defatted flaxseed hydrolysate			
100K permeate	30.0	52.3	45.6
100K retentate	78.6	37.9	48.8
10K permeate	35.7	53.5	54.8
10K retentate	45.7	35.1	38.1

Table 6. PrepLC 4000 Gradient profile

Time (min)	%A	%B	Flow rate (ml/min)
0	100	0	50
10	100	0	50
188	55	45	50
198	100	0	50
208	100	0	50
211	100	0	0

Table 7. Identity of the two most potent ACE inhibitory peptides from the defatted canola alcalase 2.4 hydrolysate

	F ₁₃₋₁₁	F ₁₈₋₅
Sequence	Val-Ser-Val	Phe-Leu
Determined MW	303.21	278.16
Calculated MW	303.18	278.16
IC ₅₀ (µg/ml)	0.44	0.37